

TITLE

## PLANT LIPOXYGENASES

This application is a continuation-in-part of U.S Application No. 09/501,422, filed February 09, 2000, which claims the benefit of U.S. Provisional Application No. 60/119,597, filed February 10, 1999. The entire content of these applications is herein incorporated by reference.

FIELD OF THE INVENTION

This invention is in the field of plant molecular biology. More specifically, this invention pertains to nucleic acid fragments encoding lipoxygenases in plants and seeds.

BACKGROUND OF THE INVENTION

Lipoxygenases are membrane-bound ubiquitous enzymes which catalyze the hydroperoxidation of polyunsaturated fatty acids in the first step of fatty acid metabolite synthesis. Products of this pathway are found as signal molecules, involved in growth and development regulation, in senescence, and in response to pathogen invasion and wound stress (Rosahl (1996) *Z. Naturforsch. [C]* 51:123-138). Lipoxygenases with different specificities, subcellular location, and tissue-specific expression patterns have been identified in several plants including rice, barley, soybean, tomato, cucumber and potato.

Many lipoxygenase cDNAs have been identified in barley, but two isozymes have been characterized, lipoxygenase 1 and 2. The amino acid sequence of proteolytic fragments from the barley isozyme 1 are identical to the predicted partial amino acid sequences from the potato loxA and the rice L2. The methyljasmonate-induced 100 kDa lipoxygenase has been characterized as a barley isozyme 2 (Voros et al. (1998) *Eur. J. Biochem.* 25:36-44). As a response to wounding, the potato Lox1 is expressed mainly in tubers and roots while Lox2 is expressed almost exclusively in leaves and Lox3 in leaves and roots. Linoleic acid is preferentially utilized as a substrate by Lox1 which produces mainly 9-hydroperoxides. Utilizing the same substrate, the major product of Lox2 and Lox3 is the jasmonic acid precursor 13-hydroperoxilinolenic acid (Royo et al. (1996) *J. Biol. Chem.* 271:1012-1019). Fungal infection of plants has allowed the identification of lipoxygenases which are induced upon microbial treatment such as the rice lipoxygenase L2 which introduces a molecular oxygen into the C-13 position of linoleic and linolenic acid (Peng et al. (1994) *J. Biol. Chem.* 269:3755-3761).

Because lipoxygenases play so many different roles and have different specificities, knowing the amino acid sequences for lipoxygenases present in

different plants will allow the understanding of plant development and wound response.

### SUMMARY OF THE INVENTION

5 The present invention concerns isolated polynucleotides comprising a nucleotide sequence encoding a polypeptide having lipoxygenase activity wherein the amino acid sequence of the polypeptide and the amino acid sequence of SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, or 18 have at least 80% sequence identity. It is preferred that the identity be at least 85%, it is preferable if the identity is at least 90%, it is more preferred that the identity be at least 95%. The present invention  
10 also relates to isolated polynucleotides comprising the complement of the nucleotide sequence, wherein the complement and the nucleotide sequence contain the same number of nucleotides and are 100% complementary. More specifically, the present invention concerns isolated polynucleotides encoding the polypeptide sequence of SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, or 18 or nucleotide sequences comprising the  
15 nucleotide sequence of SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, or 17.

In a first embodiment, the present invention concerns an isolated polynucleotide comprising: (a) a nucleotide sequence encoding a polypeptide comprising at least 100 amino acids, wherein the amino acid sequence of the polypeptide and the amino acid sequence of SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, or  
20 18 have at least 80%, 85%, 90%, or 95% identity based on the Clustal alignment method, or (b) the complement of the nucleotide sequence, wherein the complement and the nucleotide sequence contain the same number of nucleotides and are 100% complementary. The polypeptide preferably comprises the amino acid sequence of SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, or 18. The nucleotide  
25 sequence preferably comprises the nucleotide sequence of SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, or 17. The polypeptide preferably is a lipoxygenase.

In a second embodiment, the present invention relates to a recombinant DNA construct comprising any of the isolated polynucleotides of the present invention operably linked to a regulatory sequence, and a cell, a plant, and a seed comprising  
30 the recombinant DNA construct.

In a third embodiment, the present invention concerns a vector comprising any of the isolated polynucleotides of the present invention.

In a fourth embodiment, the present invention relates to an isolated polynucleotide comprising a nucleotide sequence comprised by any of the  
35 polynucleotides of the first embodiment, wherein the nucleotide sequence contains at least 30, 40, or 60 nucleotides.

In a fifth embodiment, the present invention concerns a method for transforming a cell comprising transforming a cell with any of the isolated polynucleotides of the present invention, and the cell transformed by this method. Advantageously, the cell is eukaryotic, e.g., a yeast or plant cell, or prokaryotic, e.g., a bacterium.

In a sixth embodiment, the present invention relates to a method for producing a transgenic plant comprising transforming a plant cell with any of the isolated polynucleotides of the present invention and regenerating a plant from the transformed plant cell. The invention also concerns the transgenic plant produced by this method, and the seed obtained from this transgenic plant.

In a seventh embodiment, the present invention concerns an isolated polypeptide comprising an amino acid sequence comprising at least 100 amino acids, wherein the amino acid sequence and the amino acid sequence of SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, or 18 have at least 80%, 85%, 90%, or 95% identity based on the Clustal alignment method. The amino acid sequence preferably comprises the amino acid sequence of SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, or 18. The polypeptide preferably is a lipoxygenase.

In an eighth embodiment, the invention relates to a method for isolating a polypeptide encoded by the polynucleotide of the present invention comprising isolating the polypeptide from a cell containing a recombinant DNA construct comprising the polynucleotide operably linked to a regulatory sequence.

In a ninth embodiment, the present invention concerns a virus, preferably a baculovirus, comprising any of the isolated polynucleotides of the present invention or any of the recombinant DNA constructs of the present invention.

In a tenth embodiment, the invention relates to a method of selecting an isolated polynucleotide that affects the level of expression or activity of a gene encoding a lipoxygenase protein in a host cell, preferably a plant cell, the method comprising the steps of: (a) constructing an isolated polynucleotide of the present invention or an isolated recombinant DNA construct of the present invention; (b) introducing the isolated polynucleotide or the isolated recombinant DNA construct into a host cell; (c) measuring the level of expression or activity of the lipoxygenase in the host cell containing the isolated polynucleotide; and (d) comparing the level of expression or activity of the lipoxygenase in the host cell containing the isolated polynucleotide with the level of expression or activity of the lipoxygenase in the host cell that does not contain the isolated polynucleotide.

In an eleventh embodiment, the invention concerns a method of obtaining a nucleic acid fragment encoding a substantial portion of a lipoxygenase protein,

preferably a plant lipoxygenase protein comprising the steps of: synthesizing an oligonucleotide primer comprising a nucleotide sequence of at least 30 (preferably at least 40, most preferably at least 60) contiguous nucleotides derived from a nucleotide sequence selected from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, or 17, and the complement of such nucleotide sequences; and amplifying a nucleic acid fragment (preferably a cDNA inserted in a cloning vector) using the oligonucleotide primer. The amplified nucleic acid fragment preferably will encode a substantial portion of a lipoxygenase protein amino acid sequence.

In a twelfth embodiment, this invention relates to a method of obtaining a nucleic acid fragment encoding all or a substantial portion of the amino acid sequence encoding a lipoxygenase protein comprising the steps of: probing a cDNA or genomic library with an isolated polynucleotide of the present invention; identifying a DNA clone that hybridizes with an isolated polynucleotide of the present invention; isolating the identified DNA clone; and sequencing the cDNA or genomic fragment that comprises the isolated DNA clone.

In a fourteenth embodiment, this invention relates to a method of altering the level of expression or activity of a lipoxygenase protein in a host cell comprising: (a) transforming a host cell with a recombinant DNA construct of the present invention; and (b) growing the transformed host cell under conditions that are suitable for expression of the recombinant DNA construct wherein expression of the recombinant DNA construct results in production of altered levels of the lipoxygenase protein in the transformed host cell.

#### BRIEF DESCRIPTION OF THE SEQUENCE LISTINGS

The invention can be more fully understood from the following detailed description and the accompanying Sequence Listing which form a part of this application.

Table 1 lists the polypeptides that are described herein, the designation of the cDNA clones that comprise the nucleic acid fragments encoding polypeptides representing all or a substantial portion of these polypeptides, and the corresponding identifier (SEQ ID NO:) as used in the attached Sequence Listing. The sequence descriptions and Sequence Listing attached hereto comply with the rules governing nucleotide and/or amino acid sequence disclosures in patent applications as set forth in 37 C.F.R. §1.821-1.825.

**TABLE 1**  
**Lipoxygenase Isozymes**

Protein	Clone Designation	SEQ ID NO:	
		(Nucleotide)	(Amino Acid)
Balsam Pear Lipoxygenase Isozyme	fds.pk0001.b6	1	2
Garden Balsam Lipoxygenase Isozyme	ids.pk0019.d2	3	4
Corn Lipoxygenase Isozyme	Contig of: csc1c.pk001.d6 p0126.cnlau43r	5	6
Corn Lipoxygenase Isozyme	p0118.chsbc64r	7	8
Corn Lipoxygenase Isozyme	p0128.cpicn02r	9	10
Balsam Pear Lipoxygenase Isozyme	fds.pk0001.b6:fis	11	12
Garden Balsam Lipoxygenase Isozyme	ids.pk0019.d2:fis	13	14
Corn Lipoxygenase Isozyme	p0118.chsbc64r:fis	15	16
Corn Lipoxygenase Isozyme	p0126.cnlau43r:fis	17	18
Lipoxygenase Domain I			19
Lipoxygenase Domain II			20
Lipoxygenase Domain III			21

The Sequence Listing contains the one letter code for nucleotide sequence characters and the three letter codes for amino acids as defined in conformity with the IUPAC-IUBMB standards described in *Nucleic Acids Res.* 13:3021-3030 (1985) and in the *Biochemical J.* 219 (No. 2):345-373 (1984) which are herein incorporated by reference. The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

#### DETAILED DESCRIPTION OF THE INVENTION

In the context of this disclosure, a number of terms shall be utilized. The terms "polynucleotide", "polynucleotide sequence", "nucleic acid sequence", and "nucleic acid fragment"/"isolated nucleic acid fragment" are used interchangeably herein. These terms encompass nucleotide sequences and the like. A polynucleotide may be a polymer of RNA or DNA that is single- or double-stranded, that optionally contains synthetic, non-natural or altered nucleotide bases. A polynucleotide in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA, synthetic DNA, or mixtures thereof. An isolated polynucleotide of the present invention may include at least 30 contiguous



nucleotides, preferably at least 40 contiguous nucleotides, most preferably at least 60 contiguous nucleotides derived from SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, or 17, or the complement of such sequences.

5 The term "isolated" refers to materials, such as nucleic acid molecules and/or proteins, which are substantially free or otherwise removed from components that normally accompany or interact with the materials in a naturally occurring environment. Isolated polynucleotides may be purified from a host cell in which they naturally occur. Conventional nucleic acid purification methods known to skilled artisans may be used to obtain isolated polynucleotides. The term also  
10 embraces recombinant polynucleotides and chemically synthesized polynucleotides.

20 The term "recombinant" means, for example, that a nucleic acid sequence is made by an artificial combination of two otherwise separated segments of sequence, e.g., by chemical synthesis or by the manipulation of isolated nucleic acids by genetic engineering techniques. A "recombinant DNA construct" comprises any of the isolated polynucleotides of the present invention operably linked to a regulatory sequence.

25 As used herein, "contig" refers to a nucleotide sequence that is assembled from two or more constituent nucleotide sequences that share common or overlapping regions of sequence homology. For example, the nucleotide sequences of two or more nucleic acid fragments can be compared and aligned in order to identify common or overlapping sequences. Where common or overlapping sequences exist between two or more nucleic acid fragments, the sequences (and thus their corresponding nucleic acid fragments) can be assembled  
30 into a single contiguous nucleotide sequence.

35 As used herein, "substantially similar" refers to nucleic acid fragments wherein changes in one or more nucleotide bases results in substitution of one or more amino acids, but do not affect the functional properties of the polypeptide encoded by the nucleotide sequence. "Substantially similar" also refers to nucleic acid fragments wherein changes in one or more nucleotide bases does not affect the ability of the nucleic acid fragment to mediate alteration of gene expression by gene silencing through for example antisense or co-suppression technology. "Substantially similar" also refers to modifications of the nucleic acid fragments of the instant invention such as deletion or insertion of one or more nucleotides that do not substantially affect the functional properties of the resulting transcript vis-à-vis the ability to mediate gene silencing or alteration of the functional properties of the resulting protein molecule. It is therefore understood that the invention

encompasses more than the specific exemplary nucleotide or amino acid sequences and includes functional equivalents thereof. The terms "substantially similar" and "corresponding substantially" are used interchangeably herein.

Substantially similar nucleic acid fragments may be selected by screening nucleic acid fragments representing subfragments or modifications of the nucleic acid fragments of the instant invention, wherein one or more nucleotides are substituted, deleted and/or inserted, for their ability to affect the level of the polypeptide encoded by the unmodified nucleic acid fragment in a plant or plant cell. For example, a substantially similar nucleic acid fragment representing at least 30 contiguous nucleotides, preferably at least 40 contiguous nucleotides, most preferably at least 60 contiguous nucleotides derived from the instant nucleic acid fragment can be constructed and introduced into a plant or plant cell. The level of the polypeptide encoded by the unmodified nucleic acid fragment present in a plant or plant cell exposed to the substantially similar nucleic fragment can then be compared to the level of the polypeptide in a plant or plant cell that is not exposed to the substantially similar nucleic acid fragment.

For example, it is well known in the art that antisense suppression and co-suppression of gene expression may be accomplished using nucleic acid fragments representing less than the entire coding region of a gene, and by using nucleic acid fragments that do not share 100% sequence identity with the gene to be suppressed. Moreover, alterations in a nucleic acid fragment which result in the production of a chemically equivalent amino acid at a given site, but do not effect the functional properties of the encoded polypeptide, are well known in the art. Thus, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, can also be expected to produce a functionally equivalent product. Nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the polypeptide molecule would also not be expected to alter the activity of the polypeptide. Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity of the encoded products. Consequently, an isolated polynucleotide comprising a nucleotide sequence of at least 30 (preferably at least 40, most preferably at least 60) contiguous nucleotides derived from a nucleotide sequence selected from SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, or 17 and the complement of such nucleotide

sequences may be used to affect the expression, activity, and/or function of a lipoxxygenase in a host cell. A method of using an isolated polynucleotide to affect the level of expression of a polypeptide in a host cell (eukaryotic, such as plant or yeast, prokaryotic such as bacterial) may comprise the steps of: constructing an isolated polynucleotide of the present invention or an isolated recombinant DNA construct of the present invention; introducing the isolated polynucleotide or the isolated recombinant DNA construct into a host cell; measuring the level of lipoxxygenase polypeptide or enzyme activity in the host cell containing the isolated polynucleotide or recombinant DNA construct; and comparing the level of lipoxxygenase polypeptide or enzyme activity in the host cell containing the isolated polynucleotide or recombinant DNA construct with the level of lipoxxygenase polypeptide or enzyme activity in a host cell that does not contain the isolated polynucleotide.

Moreover, substantially similar nucleic acid fragments may also be characterized by their ability to hybridize. Estimates of such homology are provided by either DNA-DNA or DNA-RNA hybridization under conditions of stringency as is well understood by those skilled in the art (Hames and Higgins, Eds. (1985) Nucleic Acid Hybridisation, IRL Press, Oxford, U.K.). Stringency conditions can be adjusted to screen for moderately similar fragments, such as homologous sequences from distantly related organisms, to highly similar fragments, such as genes that duplicate functional enzymes from closely related organisms. Post-hybridization washes determine stringency conditions. One set of preferred conditions uses a series of washes starting with 6X SSC, 0.5% SDS at room temperature for 15 min, then repeated with 2X SSC, 0.5% SDS at 45°C for 30 min, and then repeated twice with 0.2X SSC, 0.5% SDS at 50°C for 30 min. A more preferred set of stringent conditions uses higher temperatures in which the washes are identical to those above except for the temperature of the final two 30 min washes in 0.2X SSC, 0.5% SDS was increased to 60°C. Another preferred set of highly stringent conditions uses two final washes in 0.1X SSC, 0.1% SDS at 65°C.

Substantially similar nucleic acid fragments of the instant invention may also be characterized by the percent identity of the amino acid sequences that they encode to the amino acid sequences disclosed herein, as determined by algorithms commonly employed by those skilled in this art. Suitable nucleic acid fragments (isolated polynucleotides of the present invention) encode polypeptides that are at least about 70% identical, preferably at least about 80% identical to the amino acid sequences reported herein. Preferred nucleic acid fragments encode amino acid sequences that are at least about 85% identical to the amino acid sequences



reported herein. More preferred nucleic acid fragments encode amino acid sequences that are at least about 90% identical to the amino acid sequences reported herein. Most preferred are nucleic acid fragments that encode amino acid sequences that are at least about 95% identical to the amino acid sequences reported herein. Suitable nucleic acid fragments not only have the above identities but typically encode a polypeptide having at least 50 amino acids, preferably at least 100 amino acids, more preferably at least 150 amino acids, still more preferably at least 200 amino acids, and most preferably at least 250 amino acids. Sequence alignments and percent identity calculations were performed using the Megalign program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences was performed using the Clustal method of alignment (Higgins and Sharp (1989) *CABIOS*. 5:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for pairwise alignments using the Clustal method were KTUPLE 1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5.

A "substantial portion" of an amino acid or nucleotide sequence comprises an amino acid or a nucleotide sequence that is sufficient to afford putative identification of the protein or gene that the amino acid or nucleotide sequence comprises. Amino acid and nucleotide sequences can be evaluated either manually by one skilled in the art, or by using computer-based sequence comparison and identification tools that employ algorithms such as BLAST (Basic Local Alignment Search Tool; Altschul et al. (1993) *J. Mol. Biol.* 215:403-410; see also the explanation of the BLAST algorithm on the world wide web site for the National Center for Biotechnology Information at the National Library of Medicine of the National Institutes of Health). In general, a sequence of ten or more contiguous amino acids or thirty or more contiguous nucleotides is necessary in order to putatively identify a polypeptide or nucleic acid sequence as homologous to a known protein or gene. Moreover, with respect to nucleotide sequences, gene-specific oligonucleotide probes comprising 30 or more contiguous nucleotides may be used in sequence-dependent methods of gene identification (e.g., Southern hybridization) and isolation (e.g., *in situ* hybridization of bacterial colonies or bacteriophage plaques). In addition, short oligonucleotides of 12 or more nucleotides may be used as amplification primers in PCR in order to obtain a particular nucleic acid fragment comprising the primers. Accordingly, a "substantial portion" of a nucleotide sequence comprises a nucleotide sequence that will afford specific identification and/or isolation of a nucleic acid fragment comprising the sequence. The instant specification teaches amino acid and nucleotide sequences

encoding polypeptides that comprise one or more particular plant proteins. The skilled artisan, having the benefit of the sequences as reported herein, may now use all or a substantial portion of the disclosed sequences for purposes known to those skilled in this art. Accordingly, the instant invention comprises the complete sequences as reported in the accompanying Sequence Listing, as well as substantial portions of those sequences as defined above.

"Codon degeneracy" refers to divergence in the genetic code permitting variation of the nucleotide sequence without effecting the amino acid sequence of an encoded polypeptide. Accordingly, the instant invention relates to any nucleic acid fragment comprising a nucleotide sequence that encodes all or a substantial portion of the amino acid sequences set forth herein. The skilled artisan is well aware of the "codon-bias" exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid. Therefore, when synthesizing a nucleic acid fragment for improved expression in a host cell, it is desirable to design the nucleic acid fragment such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell.

"Synthetic nucleic acid fragments" can be assembled from oligonucleotide building blocks that are chemically synthesized using procedures known to those skilled in the art. These building blocks are ligated and annealed to form larger nucleic acid fragments which may then be enzymatically assembled to construct the entire desired nucleic acid fragment. "Chemically synthesized", as related to a nucleic acid fragment, means that the component nucleotides were assembled *in vitro*. Manual chemical synthesis of nucleic acid fragments may be accomplished using well established procedures, or automated chemical synthesis can be performed using one of a number of commercially available machines. Accordingly, the nucleic acid fragments can be tailored for optimal gene expression based on optimization of the nucleotide sequence to reflect the codon bias of the host cell. The skilled artisan appreciates the likelihood of successful gene expression if codon usage is biased towards those codons favored by the host. Determination of preferred codons can be based on a survey of genes derived from the host cell where sequence information is available.

"Gene" refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory

sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. "Endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "foreign-gene" refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, recombinant DNA constructs, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure.

"Coding sequence" refers to a nucleotide sequence that codes for a specific amino acid sequence. "Regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence.

Regulatory sequences may include promoters, translation leader sequences, introns, and polyadenylation recognition sequences.

"Promoter" refers to a nucleotide sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. The promoter sequence consists of proximal and more distal upstream elements, the latter elements often referred to as enhancers. Accordingly, an "enhancer" is a nucleotide sequence which can stimulate promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue-specificity of a promoter. Promoters may be derived in their entirety from a native gene, or may be composed of different elements derived from different promoters found in nature, or may even comprise synthetic nucleotide segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. Promoters which cause a nucleic acid fragment to be expressed in most cell types at most times are commonly referred to as "constitutive promoters". New promoters of various types useful in plant cells are constantly being discovered; numerous examples may be found in the compilation by Okamuro and Goldberg (1989) *Biochemistry of Plants* 15:1-82. It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, nucleic acid fragments of different lengths may have identical promoter activity.

“Translation leader sequence” refers to a nucleotide sequence located between the promoter sequence of a gene and the coding sequence. The translation leader sequence is present in the fully processed mRNA upstream of the translation start sequence. The translation leader sequence may affect processing of the primary transcript to mRNA, mRNA stability or translation efficiency. Examples of translation leader sequences have been described (Turner and Foster (1995) *Mol. Biotechnol.* 3:225-236).

“3' non-coding sequences” refer to nucleotide sequences located downstream of a coding sequence and include polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor. The use of different 3' non-coding sequences is exemplified by Ingelbrecht et al. (1989) *Plant Cell* 1:671-680.

“RNA transcript” refers to the product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. When the RNA transcript is a perfect complementary copy of the DNA sequence, it is referred to as the primary transcript or it may be a RNA sequence derived from posttranscriptional processing of the primary transcript and is referred to as the mature RNA. “Messenger RNA (mRNA)” refers to the RNA that is without introns and that can be translated into polypeptides by the cell. “cDNA” refers to DNA that is complementary to and derived from an mRNA template. The cDNA can be single-stranded or converted to double stranded form using, for example, the Klenow fragment of DNA polymerase I. “Sense-RNA” refers to an RNA transcript that includes the mRNA and so can be translated into a polypeptide by the cell. “Antisense RNA” refers to an RNA transcript that is complementary to all or part of a target primary transcript or mRNA and that blocks the expression of a target gene (see U.S. Patent No. 5,107,065, incorporated herein by reference). The complementarity of an antisense RNA may be with any part of the specific nucleotide sequence, i.e., at the 5' non-coding sequence, 3' non-coding sequence, introns, or the coding sequence. “Functional RNA” refers to sense RNA, antisense RNA, ribozyme RNA, or other RNA that may not be translated but yet has an effect on cellular processes.

The term “operably linked” refers to the association of two or more nucleic acid fragments on a single polynucleotide so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (i.e., that the coding



sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

The term "expression", as used herein, refers to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid fragment of the invention. Expression may also refer to translation of mRNA into a polypeptide. "Antisense inhibition" refers to the production of antisense RNA transcripts capable of suppressing the expression of the target protein.

"Overexpression" refers to the production of a gene product in transgenic organisms that exceeds levels of production in normal or non-transformed organisms.

"Co-suppression" refers to the production of sense RNA transcripts capable of suppressing the expression of identical or substantially similar foreign or endogenous genes (U.S. Patent No. 5,231,020, incorporated herein by reference).

A "protein" or "polypeptide" is a chain of amino acids arranged in a specific order determined by the coding sequence in a polynucleotide encoding the polypeptide. Each protein or polypeptide has a unique function.

"Altered levels" or "altered expression" refers to the production of gene product(s) in transgenic organisms in amounts or proportions that differ from that of normal or non-transformed organisms.

"Mature protein" or the term "mature" when used in describing a protein refers to a post-translationally processed polypeptide; i.e., one from which any pre- or propeptides present in the primary translation product have been removed.

"Precursor protein" or the term "precursor" when used in describing a protein refers to the primary product of translation of mRNA; i.e., with pre- and propeptides still present. Pre- and propeptides may be but are not limited to intracellular localization signals.

A "chloroplast transit peptide" is an amino acid sequence which is translated in conjunction with a protein and directs the protein to the chloroplast or other plastid types present in the cell in which the protein is made. "Chloroplast transit sequence" refers to a nucleotide sequence that encodes a chloroplast transit peptide. A "signal peptide" is an amino acid sequence which is translated in conjunction with a protein and directs the protein to the secretory system (Chrispeels (1991) *Ann. Rev. Plant Phys. Plant Mol. Biol.* 42:21-53). If the protein is to be directed to a vacuole, a vacuolar targeting signal (*supra*) can further be added, or if to the endoplasmic reticulum, an endoplasmic reticulum retention signal (*supra*) may be added. If the protein is to be directed to the nucleus, any signal peptide present should be removed and instead a nuclear localization signal included (Raikhel (1992) *Plant Phys.* 100:1627-1632).

“Transformation” refers to the transfer of a nucleic acid fragment into the genome of a host organism, resulting in genetically stable inheritance. Host organisms containing the transformed nucleic acid fragments are referred to as “transgenic” organisms. Examples of methods of plant transformation include

5 *Agrobacterium*-mediated transformation (De Blaere et al. (1987) *Meth. Enzymol.* 143:277) and particle-accelerated or “gene gun” transformation technology (Klein et al. (1987) *Nature (London)* 327:70-73; U.S. Patent No. 4,945,050, incorporated herein by reference). Thus, isolated polynucleotides of the present invention can be incorporated into recombinant constructs, typically DNA constructs, capable of

10 introduction into and replication in a host cell. Such a construct can be a vector that includes a replication system and sequences that are capable of transcription and translation of a polypeptide-encoding sequence in a given host cell. A number of vectors suitable for stable transfection of plant cells or for the establishment of transgenic plants have been described in, e.g., Pouwels et al., *Cloning Vectors: A Laboratory Manual*, 1985, supp. 1987; Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academic Press, 1989; and Flevin et al., *Plant Molecular Biology Manual*, Kluwer Academic Publishers, 1990. Typically, plant expression vectors include, for example, one or more cloned plant genes under the transcriptional control of 5' and 3' regulatory sequences and a dominant selectable

15 marker. Such plant expression vectors also can contain a promoter regulatory region (e.g., a regulatory region controlling inducible or constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

25 Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described more fully in Sambrook et al. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, 1989 (hereinafter “Maniatis”).

30 “PCR” or “polymerase chain reaction” is well known by those skilled in the art as a technique used for the amplification of specific DNA segments (U.S. Patent Nos. 4,683,195 and 4,800,159).

The present invention concerns an isolated polynucleotide comprising a nucleotide sequence encoding a lipoxygenase polypeptide having at least 80% identity, based on the Clustal method of alignment, when compared to a polypeptide

35 of SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, or 18.

This invention also relates to the isolated complement of such polynucleotides, wherein the complement and the polynucleotide consist of the

same number of nucleotides, and the nucleotide sequences of the complement and the polynucleotide have 100% complementarity.

Nucleic acid fragments encoding at least a portion of several lipoxygenases have been isolated and identified by comparison of random plant cDNA sequences to public databases containing nucleotide and protein sequences using the BLAST algorithms well known to those skilled in the art. The nucleic acid fragments of the instant invention may be used to isolate cDNAs and genes encoding homologous proteins from the same or other plant species. Isolation of homologous genes using sequence-dependent protocols is well known in the art. Examples of sequence-dependent protocols include, but are not limited to, methods of nucleic acid hybridization, and methods of DNA and RNA amplification as exemplified by various uses of nucleic acid amplification technologies (e.g., polymerase chain reaction, ligase chain reaction).

For example, genes encoding other lipoxygenases, either as cDNAs or genomic DNAs, could be isolated directly by using all or a portion of the instant nucleic acid fragments as DNA hybridization probes to screen libraries from any desired plant employing methodology well known to those skilled in the art. Specific oligonucleotide probes based upon the instant nucleic acid sequences can be designed and synthesized by methods known in the art (Maniatis). Moreover, an entire sequence can be used directly to synthesize DNA probes by methods known to the skilled artisan such as random primer DNA labeling, nick translation, end-labeling techniques, or RNA probes using available *in vitro* transcription systems. In addition, specific primers can be designed and used to amplify a part or all of the instant sequences. The resulting amplification products can be labeled directly during amplification reactions or labeled after amplification reactions, and used as probes to isolate full length cDNA or genomic fragments under conditions of appropriate stringency.

In addition, two short segments of the instant nucleic acid fragments may be used in polymerase chain reaction protocols to amplify longer nucleic acid fragments encoding homologous genes from DNA or RNA. The polymerase chain reaction may also be performed on a library of cloned nucleic acid fragments wherein the sequence of one primer is derived from the instant nucleic acid fragments, and the sequence of the other primer takes advantage of the presence of the polyadenylic acid tracts to the 3' end of the mRNA precursor encoding plant genes. Alternatively, the second primer sequence may be based upon sequences derived from the cloning vector. For example, the skilled artisan can follow the RACE protocol (Frohman et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:8998-9002) to

generate cDNAs by using PCR to amplify copies of the region between a single point in the transcript and the 3' or 5' end. Primers oriented in the 3' and 5' directions can be designed from the instant sequences. Using commercially available 3' RACE or 5' RACE systems (BRL), specific 3' or 5' cDNA fragments can be isolated (Ohara et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:5673-5677; Loh et al. (1989) *Science* 243:217-220). Products generated by the 3' and 5' RACE procedures can be combined to generate full-length cDNAs (Frohman and Martin (1989) *Techniques* 1:165). Consequently, a polynucleotide comprising a nucleotide sequence of at least 30 (preferably at least 40, most preferably at least 60) contiguous nucleotides derived from a nucleotide sequence of SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, or 17 and the complement of such nucleotide sequences may be used in such methods to obtain a nucleic acid fragment encoding a substantial portion of an amino acid sequence of a polypeptide.

Availability of the instant nucleotide and deduced amino acid sequences facilitates immunological screening of cDNA expression libraries. Synthetic peptides representing portions of the instant amino acid sequences may be synthesized. These peptides can be used to immunize animals to produce polyclonal or monoclonal antibodies with specificity for peptides or proteins comprising the amino acid sequences. These antibodies can be then be used to screen cDNA expression libraries to isolate full-length cDNA clones of interest (Lerner (1984) *Adv. Immunol.* 36:1-34; Maniatis).

In another embodiment, this invention concerns viruses and host cells comprising either the recombinant DNA constructs of the invention as described herein or isolated polynucleotides of the invention as described herein. Examples of host cells which can be used to practice the invention include, but are not limited to, yeast, bacteria, and plants.

The nucleic acid fragments of the instant invention may be used to create transgenic plants in which the disclosed polypeptides are present at higher or lower levels than normal or in cell types or developmental stages in which they are not normally found. This would have the effect of altering the level of pathogen resistance in those cells. Overexpression of lipoxygenase isozymes in transgenic plants will result in the production of novel oils containing fatty acids with double bounds. These novel oils can be used as drying agents and as livestock feed supplement to enhance lean body mass. Blocking the expression of the lipoxygenase gene in developing wheat kernels will result in improved stability of the grain. The protein and oil products from soybeans having suppressed lipoxygenases will have improved flavor. The lipoxygenase sequence may be used



to obtain the lox promoter(s). This promoter(s) may be used in combination with various structural gene sequences to achieve crops with improved flavor and protein functionality.

Overexpression of the proteins of the instant invention may be accomplished by first constructing a recombinant DNA construct in which the coding region is operably linked to a promoter capable of directing expression of a gene in the desired tissues at the desired stage of development. The recombinant DNA construct may comprise promoter sequences and translation leader sequences derived from the same genes. 3' Non-coding sequences encoding transcription termination signals may also be provided. The instant recombinant DNA construct may also comprise one or more introns in order to facilitate gene expression.

Plasmid vectors comprising the instant isolated polynucleotide (or recombinant DNA construct) may be constructed. The choice of plasmid vector is dependent upon the method that will be used to transform host plants. The skilled artisan is well aware of the genetic elements that must be present on the plasmid vector in order to successfully transform, select and propagate host cells containing the recombinant DNA construct or chimeric gene. The skilled artisan will also recognize that different independent transformation events will result in different levels and patterns of expression (Jones et al. (1985) *EMBO J.* 4:2411-2418; De Almeida et al. (1989) *Mol. Gen. Genetics* 218:78-86), and thus that multiple events must be screened in order to obtain lines displaying the desired expression level and pattern. Such screening may be accomplished by Southern analysis of DNA, Northern analysis of mRNA expression, Western analysis of protein expression, or phenotypic analysis.

For some applications it may be useful to direct the instant polypeptide to different cellular compartments, or to facilitate its secretion from the cell. It is thus envisioned that the recombinant DNA construct described above may be further supplemented by directing the coding sequence to encode the instant polypeptide with appropriate intracellular targeting sequences such as transit sequences (Keegstra (1989) *Cell* 56:247-253), signal sequences, or sequences encoding endoplasmic reticulum localization (Chrispeels (1991) *Ann. Rev. Plant Phys. Plant Mol. Biol.* 42:21-53) or nuclear localization signals (Raikhel (1992) *Plant Phys.* 100:1627-1632) with or without removing targeting sequences that are already present. While the references cited give examples of each of these, the list is not exhaustive and more targeting signals of use may be discovered in the future.

It may also be desirable to reduce or eliminate expression of genes encoding the instant polypeptides in plants for some applications. In order to accomplish this,

a recombinant DNA construct designed for co-suppression of the instant polypeptide can be constructed by linking a gene or gene fragment encoding that polypeptide to plant promoter sequences. Alternatively, a recombinant DNA construct designed to express antisense RNA for all or part of the instant nucleic acid fragment can be constructed by linking the gene or gene fragment in reverse orientation to plant promoter sequences. Either the co-suppression or antisense recombinant DNA constructs could be introduced into plants via transformation wherein expression of the corresponding endogenous genes are reduced or eliminated.

Molecular genetic solutions to the generation of plants with altered gene expression have a decided advantage over more traditional plant breeding approaches. Changes in plant phenotypes can be produced by specifically inhibiting expression of one or more genes by antisense inhibition or cosuppression (U.S. Patent Nos. 5,190,931, 5,107,065 and 5,283,323). An antisense or cosuppression construct would act as a dominant negative regulator of gene activity. While conventional mutations can yield negative regulation of gene activity these effects are most likely recessive. The dominant negative regulation available with a transgenic approach may be advantageous from a breeding perspective. In addition, the ability to restrict the expression of a specific phenotype to the reproductive tissues of the plant by the use of tissue specific promoters may confer agronomic advantages relative to conventional mutations which may have an effect in all tissues in which a mutant gene is ordinarily expressed.

The person skilled in the art will know that special considerations are associated with the use of antisense or cosuppression technologies in order to reduce expression of particular genes. For example, the proper level of expression of sense or antisense genes may require the use of different recombinant DNA constructs utilizing different regulatory elements known to the skilled artisan. Once transgenic plants are obtained by one of the methods described above, it will be necessary to screen individual transgenics for those that most effectively display the desired phenotype. Accordingly, the skilled artisan will develop methods for screening large numbers of transformants. The nature of these screens will generally be chosen on practical grounds. For example, one can screen by looking for changes in gene expression by using antibodies specific for the protein encoded by the gene being suppressed, or one could establish assays that specifically measure enzyme activity. A preferred method will be one which allows large numbers of samples to be processed rapidly, since it will be expected that a large number of transformants will be negative for the desired phenotype.

In another embodiment, the present invention concerns a lipoxygenase polypeptide having an amino acid sequence that is at least 80% identical, based on the Clustal method of alignment, to a polypeptide of SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, or 18.

5 The instant polypeptides (or portions thereof) may be produced in heterologous host cells, particularly in the cells of microbial hosts, and can be used to prepare antibodies to these proteins by methods well known to those skilled in the art. The antibodies are useful for detecting the polypeptides of the instant invention *in situ* in cells or *in vitro* in cell extracts. Preferred heterologous host cells  
10 for production of the instant polypeptides are microbial hosts. Microbial expression systems and expression vectors containing regulatory sequences that direct high level expression of foreign proteins are well known to those skilled in the art. Any of these could be used to construct a recombinant DNA construct for production of the instant polypeptides. This recombinant DNA construct could then be introduced into  
15 appropriate microorganisms via transformation to provide high level expression of the encoded lipoxygenases. An example of a vector for high level expression of the instant polypeptides in a bacterial host is provided (Example 6).

All or a substantial portion of the polynucleotides of the instant invention may also be used as probes for genetically and physically mapping the genes that they  
20 are a part of, and used as markers for traits linked to those genes. Such information may be useful in plant breeding in order to develop lines with desired phenotypes. For example, the instant nucleic acid fragments may be used as restriction fragment length polymorphism (RFLP) markers. Southern blots (Maniatis) of restriction-digested plant genomic DNA may be probed with the nucleic  
25 acid fragments of the instant invention. The resulting banding patterns may then be subjected to genetic analyses using computer programs such as MapMaker (Lander et al. (1987) *Genomics* 1:174-181) in order to construct a genetic map. In addition, the nucleic acid fragments of the instant invention may be used to probe Southern blots containing restriction endonuclease-treated genomic DNAs of a set of  
30 individuals representing parent and progeny of a defined genetic cross. Segregation of the DNA polymorphisms is noted and used to calculate the position of the instant nucleic acid sequence in the genetic map previously obtained using this population (Botstein et al. (1980) *Am. J. Hum. Genet.* 32:314-331).

The production and use of plant gene-derived probes for use in genetic  
35 mapping is described in Bernatzky and Tanksley (1986) *Plant Mol. Biol. Reporter* 4:37-41. Numerous publications describe genetic mapping of specific cDNA clones using the methodology outlined above or variations thereof. For example, F2

intercross populations, backcross populations, randomly mated populations, near isogenic lines, and other sets of individuals may be used for mapping. Such methodologies are well known to those skilled in the art.

5 Nucleic acid probes derived from the instant nucleic acid sequences may also be used for physical mapping (i.e., placement of sequences on physical maps; see Hoheisel et al. In: *Nonmammalian Genomic Analysis: A Practical Guide*, Academic press 1996, pp. 319-346, and references cited therein).

10 Nucleic acid probes derived from the instant nucleic acid sequences may be used in direct fluorescence *in situ* hybridization (FISH) mapping (Trask (1991) *Trends Genet.* 7:149-154). Although current methods of FISH mapping favor use of large clones (several kb to several hundred kb; see Laan et al. (1995) *Genome Res.* 5:13-20), improvements in sensitivity may allow performance of FISH mapping using shorter probes.

15 A variety of nucleic acid amplification-based methods of genetic and physical mapping may be carried out using the instant nucleic acid sequences. Examples include allele-specific amplification (Kazazian (1989) *J. Lab. Clin. Med.* 11:95-96), polymorphism of PCR-amplified fragments (CAPS; Sheffield et al. (1993) *Genomics* 16:325-332), allele-specific ligation (Landegren et al. (1988) *Science* 241:1077-1080), nucleotide extension reactions (Sokolov (1990) *Nucleic Acid Res.* 18:3671), Radiation Hybrid Mapping (Walter et al. (1997) *Nat. Genet.* 7:22-28) and Happy Mapping (Dear and Cook (1989) *Nucleic Acid Res.* 17:6795-6807). For these methods, the sequence of a nucleic acid fragment is used to design and produce primer pairs for use in the amplification reaction or in primer extension reactions. The design of such primers is well known to those skilled in the art. In  
20 methods employing PCR-based genetic mapping, it may be necessary to identify DNA sequence differences between the parents of the mapping cross in the region corresponding to the instant nucleic acid sequence. This, however, is generally not necessary for mapping methods.

25 Loss of function mutant phenotypes may be identified for the instant cDNA clones either by targeted gene disruption protocols or by identifying specific mutants for these genes contained in a maize population carrying mutations in all possible genes (Ballinger and Benzer (1989) *Proc. Natl. Acad. Sci. USA* 86:9402-9406; Koes et al. (1995) *Proc. Natl. Acad. Sci. USA* 92:8149-8153; Bensen et al. (1995) *Plant Cell* 7:75-84). The latter approach may be accomplished in two ways. First, short  
35 segments of the instant nucleic acid fragments may be used in polymerase chain reaction protocols in conjunction with a mutation tag sequence primer on DNAs prepared from a population of plants in which Mutator transposons or some other



mutation-causing DNA element has been introduced (see Bensen, *supra*). The amplification of a specific DNA fragment with these primers indicates the insertion of the mutation tag element in or near the plant gene encoding the instant polypeptides. Alternatively, the instant nucleic acid fragment may be used as a hybridization probe against PCR amplification products generated from the mutation population using the mutation tag sequence primer in conjunction with an arbitrary genomic site primer, such as that for a restriction enzyme site-anchored synthetic adaptor. With either method, a plant containing a mutation in the endogenous gene encoding the instant polypeptides can be identified and obtained. This mutant plant can then be used to determine or confirm the natural function of the instant polypeptides disclosed herein.

### EXAMPLES

The present invention is further defined in the following Examples, in which parts and percentages are by weight and degrees are Celsius, unless otherwise stated. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, various modifications of the invention in addition to those shown and described herein will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

The disclosure of each reference set forth herein is incorporated herein by reference in its entirety.

### EXAMPLE 1

#### Composition of cDNA Libraries; Isolation and Sequencing of cDNA Clones

cDNA libraries representing mRNAs from various balsam pear, garden balsam, and corn tissues were prepared. The characteristics of the libraries are described below.



templates are reacted with vector primed M13 forward and reverse oligonucleotides in a PCR-based sequencing reaction and loaded onto automated sequencers. Confirmation of clone identification is performed by sequence alignment to the original EST sequence from which the FIS request is made.

Confirmed templates are transposed via the Primer Island transposition kit (PE Applied Biosystems, Foster City, CA) which is based upon the *Saccharomyces cerevisiae* Ty1 transposable element (Devine and Boeke (1994) *Nucleic Acids Res.* 22:3765-3772). The *in vitro* transposition system places unique binding sites randomly throughout a population of large DNA molecules. The transposed DNA is then used to transform DH10B electro-competent cells (Gibco BRL/Life Technologies, Rockville, MD) via electroporation. The transposable element contains an additional selectable marker (named DHFR; Fling and Richards (1983) *Nucleic Acids Res.* 11:5147-5158), allowing for dual selection on agar plates of only those subclones containing the integrated transposon. Multiple subclones are randomly selected from each transposition reaction, plasmid DNAs are prepared via alkaline lysis, and templates are sequenced (ABI Prism dye-terminator ReadyReaction mix) outward from the transposition event site, utilizing unique primers specific to the binding sites within the transposon.

Sequence data is collected (ABI Prism Collections) and assembled using Phred/Phrap (P. Green, University of Washington, Seattle). Phred/Phrap is a public domain software program which re-reads the ABI sequence data, re-calls the bases, assigns quality values, and writes the base calls and quality values into editable output files. The Phrap sequence assembly program uses these quality values to increase the accuracy of the assembled sequence contigs. Assemblies are viewed by the Consed sequence editor (D. Gordon, University of Washington, Seattle).

In some of the clones the cDNA fragment corresponds to a portion of the 3'-terminus of the gene and does not cover the entire open reading frame. In order to obtain the upstream information one of two different protocols are used. The first of these methods results in the production of a fragment of DNA containing a portion of the desired gene sequence while the second method results in the production of a fragment containing the entire open reading frame. Both of these methods use two rounds of PCR amplification to obtain fragments from one or more libraries. The libraries some times are chosen based on previous knowledge that the specific gene should be found in a certain tissue and some times are randomly-chosen. Reactions to obtain the same gene may be performed on several libraries in parallel or on a pool of libraries. Library pools are normally prepared using from 3 to 5 different libraries and normalized to a uniform dilution. In the first round of

amplification both methods use a vector-specific (forward) primer corresponding to a portion of the vector located at the 5'-terminus of the clone coupled with a gene-specific (reverse) primer. The first method uses a sequence that is complementary to a portion of the already known gene sequence while the second method uses a gene-specific primer complementary to a portion of the 3'-untranslated region (also referred to as UTR). In the second round of amplification a nested set of primers is used for both methods. The resulting DNA fragment is ligated into a pBluescript vector using a commercial kit and following the manufacturer's protocol. This kit is selected from many available from several vendors including Invitrogen (Carlsbad, CA), Promega Biotech (Madison, WI), and Gibco-BRL (Gaithersburg, MD). The plasmid DNA is isolated by alkaline lysis method and submitted for sequencing and assembly using Phred/Phrap, as above.

## EXAMPLE 2

### Identification of cDNA Clones

cDNA clones encoding lipxygenases were identified by conducting BLAST (Basic Local Alignment Search Tool; Altschul et al. (1993) *J. Mol. Biol.* 215:403-410; see also the explanation of the BLAST algorithm on the world wide web site for the National Center for Biotechnology Information at the National Library of Medicine of the National Institutes of Health) searches for similarity to sequences contained in the BLAST "nr" database (comprising all non-redundant GenBank CDS translations, sequences derived from the 3-dimensional structure Brookhaven Protein Data Bank, the last major release of the SWISS-PROT protein sequence database, EMBL, and DDBJ databases). The cDNA sequences obtained in Example 1 were analyzed for similarity to all publicly available DNA sequences contained in the "nr" database using the BLASTN algorithm provided by the National Center for Biotechnology Information (NCBI). The DNA sequences were translated in all reading frames and compared for similarity to all publicly available protein sequences contained in the "nr" database using the BLASTX algorithm (Gish and States (1993) *Nat. Genet.* 3:266-272) provided by the NCBI. For convenience, the P-value (probability) of observing a match of a cDNA sequence to a sequence contained in the searched databases merely by chance as calculated by BLAST are reported herein as "pLog" values, which represent the negative of the logarithm of the reported P-value. Accordingly, the greater the pLog value, the greater the likelihood that the cDNA sequence and the BLAST "hit" represent homologous proteins.

ESTs submitted for analysis are compared to the genbank database as described above. ESTs that contain sequences more 5- or 3-prime can be found by



using the BLASTn algorithm (Altschul et al (1997) *Nucleic Acids Res.* 25:3389-3402.) against the Du Pont proprietary database comparing nucleotide sequences that share common or overlapping regions of sequence homology. Where common or overlapping sequences exist between two or more nucleic acid fragments, the sequences can be assembled into a single contiguous nucleotide sequence, thus extending the original fragment in either the 5 or 3 prime direction. Once the most 5-prime EST is identified, its complete sequence can be determined by Full Insert Sequencing as described in Example 1. Homologous genes belonging to different species can be found by comparing the amino acid sequence of a known gene (from either a proprietary source or a public database) against an EST database using the tBLASTn algorithm. The tBLASTn algorithm searches an amino acid query against a nucleotide database that is translated in all 6 reading frames. This search allows for differences in nucleotide codon usage between different species, and for codon degeneracy.

### EXAMPLE 3

#### Characterization of cDNA Clones Encoding Lipoxxygenase Isozymes

The BLASTX search using the EST sequences from clones listed in Table 3 revealed similarity of the polypeptides encoded by the cDNAs to lipoxxygenase isozymes from *Solanum tuberosum* (NCBI General Identifier Nos. 1495802 and 765203), the lipoxxygenase 1 from *Hordeum vulgare* or *Cucumis sativus* (NCBI General Identifier Nos. 2182267 and 3668063, respectively), or lipoxxygenase L2 from *Oryza sativa* (NCBI General Identifier No. 126401). Shown in Table 3 are the BLAST results for individual ESTs ("EST"), or for the sequences of contigs assembled from two or more ESTs ("Contig"):

TABLE 3

BLAST Results for Sequences Encoding Polypeptides  
Homologous to Lypoxxygenase Isozymes

Clone	Status	NCBI General Identifier No.	BLAST pLog Score
fds.pk0001.b6	EST	3668063	82.5
ids.pk0019.d2	EST	765203	43.1
Contig of: csc1c.pk001.d6 p0126.cnlau43r	Contig	1495802	57.2
p0118.chsbc64r	EST	2182267	25.4
p0128.cpicn02r	EST	126401	74.1

The sequence of the entire cDNA insert in clones fds.pk0001.b6, ids.pk0019.d2, p0118.chsbc64r, and p0128.cpicn02r was determined. The

BLASTP search using the amino acid sequences from clones listed in Table 4 revealed similarity of the polypeptides encoded by the cDNAs to lipoxygenase isozymes from *Solanum tuberosum* (NCBI General Identifier Nos. 1495802 and 765203), lipoxygenase 1 from *Cucumis sativus* (NCBI General Identifier No. 3668063), or lipoxygenase from *Hordeum vulgare* (NCBI General Identifier No. 2182267). Shown in Table 4 are the BLASTP results for the sequences of the entire cDNA inserts comprising the indicated cDNA clones ("FIS"), or for FISs encoding the entire protein ("CGS"):

10

**TABLE 4**  
BLAST Results for Sequences Encoding Polypeptides  
Homologous to Lipoxygenase Isozymes

Clone	Status	NCBI General Identifier No.	BLAST pLog Score
fds.pk0001.b6:fis	CGS	3668063	>254.00
ids.pk0019.d2:fis	FIS	765203	>254.00
p0118.chsbc64r:fis	CGS	2182267	>180.00
p0126.cnlau43r:fis	FIS	1495802	147.00

15

The nucleotide sequence of the entire cDNA insert in clone fds.pk0001.b6:fis is shown in SEQ ID NO:11. The amino acid sequence deduced from nucleotides 12 through 2651 of SEQ ID NO:11 is shown in SEQ ID NO:12. The nucleotide sequence of the entire cDNA insert in clone ids.pk0019.d2:fis is shown in SEQ ID NO:13. The amino acid sequence deduced from nucleotides 20 through 1336 of SEQ ID NO:13 is shown in SEQ ID NO:14. The nucleotide sequence of the entire cDNA insert in clone p0118.chsbc64r:fis is shown in SEQ ID NO:15. The amino acid sequence deduced from nucleotides 235 through 2895 of SEQ ID NO:15 is shown in SEQ ID NO:16. The nucleotide sequence of the entire cDNA insert in clone p0126.cnlau43r:fis is shown in SEQ ID NO:17. The amino acid sequence deduced from amino acids 96 through 1495 of SEQ ID NO:17 is shown in SEQ ID NO:18.

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A number of conserved regions are known among plant Lipoxygenases. A 50 amino acid stretch (SEQ ID NO:19) towards the C-terminal half of the protein contains 5 histidines conserved in lipoxigenases. A 32 amino acid stretch (SEQ ID NO:20) includes another conserved histidine. These histidines were first identified at positions 512, 517, 522, 540, 549, and 708 of the barley LoxA (Mechelen et al. (1999) *Plant Molecular Biology* 39:1283-1298). Three of these histidines (at positions 517, 522, and 708) are considered to be necessary for iron binding and catalytic activity. The C-terminal amino acids (SEQ ID NO:21) are also conserved

in lipoxygenases. The 50 amino acid domain of SEQ ID NO:19 corresponds to amino acids 532 through 581 of SEQ ID NO:12, amino acids 97 through 146 of SEQ ID NO:14, and amino acids 528 through 577 of SEQ ID NO:16. The 32 amino acid domain of SEQ ID NO:20 corresponds to amino acids 724 through 755 of SEQ ID NO:12, amino acids 289 through 320 of SEQ ID NO:14, and amino acids 720 through 751 of SEQ ID NO:16. The 8 amino acid domain of SEQ ID NO:21 corresponds to amino acids 873 through 880 of SEQ ID NO:12, and amino acids 880 through 887 of SEQ ID NO:16. In SEQ ID NO:14, the domain of SEQ ID NO:21 is found at amino acids 438 through 445, but there is a conserved change (Ile to Val) at position 443 and a non-conserved change (Pro to Ser) at position 440 of SEQ ID NO:14.

The data in Table 5 presents the percent identity of the amino acid sequences set forth in SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, and 18 with the *Cucumis sativus*, *Solanum tuberosum*, and *Hordeum vulgare* sequences (NCBI General Identifiers Nos. 3668063, 765203, 1495802, and 2182267).

**TABLE 5**  
Percent Identity of Amino Acid Sequences Deduced From the Nucleotide Sequences of cDNA Clones Encoding Polypeptides Homologous to Lipoxygenase Isozymes

	SEQ ID NO.	3668063	765203	2182267	1495802
fds.pk0001.b6	2	75.9	35.9	31.2	17.1
ids.pk0019.d2	4	62.7	69.6	49.0	36.3
Contig of: csc1c.pk001.d6 p0126.cnlau43r	6	33.3	32.7	27.2	57.8
p0118.chsbc64r	8	22.5	22.5	61.3	16.2
p0128.cpicn02r	10	38.9	42.7	37.4	16.8
fds.pk0001.b6:fis	12	81.7	64.9	48.9	38.2
ids.pk0019.d2:fis	14	65.6	72.6	59.3	47.2
p0118.chsbc64r:fis	16	49.4	56.0	71.1	33.8
p0126.cnlau43r:fis	18	32.1	33.2	31.0	53.0

Sequence alignments and percent identity calculations were performed using the Megalign program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences was performed using the Clustal method of alignment (Higgins and Sharp (1989) *CABIOS* 5:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for pairwise alignments using the Clustal

method were KTUPLE 1, GAP PENALTY=3, WINDOW=5 and DIAGONALS  
SAVED=5. Sequence alignments and BLAST scores and probabilities indicate that  
the nucleic acid fragments comprising the instant cDNA clones encode a substantial  
portion or entire lipoxygenase isozymes from balsam pear, garden balsam, and  
5 corn. These sequences represent the first balsam pear, garden balsam, and corn  
sequences encoding lipoxygenase isozymes known to Applicant.

#### EXAMPLE 4

##### Expression of Recombinant DNA Constructs in Monocot Cells

10 A recombinant DNA construct comprising a cDNA encoding the instant  
polypeptides in sense orientation with respect to the maize 27 kD zein promoter that  
is located 5' to the cDNA fragment, and the 10 kD zein 3' end that is located 3' to  
the cDNA fragment, can be constructed. The cDNA fragment of this gene may be  
generated by polymerase chain reaction (PCR) of the cDNA clone using appropriate  
oligonucleotide primers. Cloning sites (NcoI or SmaI) can be incorporated into the  
15 oligonucleotides to provide proper orientation of the DNA fragment when inserted  
into the digested vector pML103 as described below. Amplification is then  
performed in a standard PCR. The amplified DNA is then digested with restriction  
enzymes NcoI and SmaI and fractionated on an agarose gel. The appropriate band  
can be isolated from the gel and combined with a 4.9 kb NcoI-SmaI fragment of the  
20 plasmid pML103. Plasmid pML103 has been deposited under the terms of the  
Budapest Treaty at ATCC (American Type Culture Collection, 10801 University  
Blvd., Manassas, VA 20110-2209), and bears accession number ATCC 97366. The  
DNA segment from pML103 contains a 1.05 kb Sall-NcoI promoter fragment of the  
maize 27 kD zein gene and a 0.96 kb SmaI-Sall fragment from the 3' end of the  
25 maize 10 kD zein gene in the vector pGem9Zf(+) (Promega). Vector and insert  
DNA can be ligated at 15°C overnight, essentially as described (Maniatis). The  
ligated DNA may then be used to transform *E. coli* XL1-Blue (Epicurian Coli XL-1  
Blue™; Stratagene). Bacterial transformants can be screened by restriction enzyme  
digestion of plasmid DNA and limited nucleotide sequence analysis using the  
30 dideoxy chain termination method (Sequenase™ DNA Sequencing Kit; U.S.  
Biochemical). The resulting plasmid construct would comprise a recombinant DNA  
construct encoding, in the 5' to 3' direction, the maize 27 kD zein promoter, a cDNA  
fragment encoding the instant polypeptides, and the 10 kD zein 3' region.

35 The recombinant DNA construct described above can then be introduced into  
corn cells by the following procedure. Immature corn embryos can be dissected  
from developing caryopses derived from crosses of the inbred corn lines H99 and  
LH132. The embryos are isolated 10 to 11 days after pollination when they are 1.0



to 1.5 mm long. The embryos are then placed with the axis-side facing down and in contact with agarose-solidified N6 medium (Chu et al. (1975) *Sci. Sin. Peking* 18:659-668). The embryos are kept in the dark at 27°C. Friable embryogenic callus consisting of undifferentiated masses of cells with somatic proembryoids and embryoids borne on suspensor structures proliferates from the scutellum of these immature embryos. The embryogenic callus isolated from the primary explant can be cultured on N6 medium and sub-cultured on this medium every 2 to 3 weeks.

The plasmid, p35S/Ac (obtained from Dr. Peter Eckes, Hoechst Ag, Frankfurt, Germany) may be used in transformation experiments in order to provide for a selectable marker. This plasmid contains the *Pat* gene (see European Patent Publication 0 242 236) which encodes phosphinothricin acetyl transferase (PAT). The enzyme PAT confers resistance to herbicidal glutamine synthetase inhibitors such as phosphinothricin. The *pat* gene in p35S/Ac is under the control of the 35S promoter from Cauliflower Mosaic Virus (Odell et al. (1985) *Nature* 313:810-812) and the 3' region of the nopaline synthase gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*.

The particle bombardment method (Klein et al. (1987) *Nature* 327:70-73) may be used to transfer genes to the callus culture cells. According to this method, gold particles (1  $\mu\text{m}$  in diameter) are coated with DNA using the following technique. Ten  $\mu\text{g}$  of plasmid DNAs are added to 50  $\mu\text{L}$  of a suspension of gold particles (60 mg per mL). Calcium chloride (50  $\mu\text{L}$  of a 2.5 M solution) and spermidine free base (20  $\mu\text{L}$  of a 1.0 M solution) are added to the particles. The suspension is vortexed during the addition of these solutions. After 10 minutes, the tubes are briefly centrifuged (5 sec at 15,000 rpm) and the supernatant removed. The particles are resuspended in 200  $\mu\text{L}$  of absolute ethanol, centrifuged again and the supernatant removed. The ethanol rinse is performed again and the particles resuspended in a final volume of 30  $\mu\text{L}$  of ethanol. An aliquot (5  $\mu\text{L}$ ) of the DNA-coated gold particles can be placed in the center of a Kapton™ flying disc (Bio-Rad Labs). The particles are then accelerated into the corn tissue with a Biolistic™ PDS-1000/He (Bio-Rad Instruments, Hercules CA), using a helium pressure of 1000 psi, a gap distance of 0.5 cm and a flying distance of 1.0 cm.

For bombardment, the embryogenic tissue is placed on filter paper over agarose-solidified N6 medium. The tissue is arranged as a thin lawn and covered a circular area of about 5 cm in diameter. The petri dish containing the tissue can be placed in the chamber of the PDS-1000/He approximately 8 cm from the stopping screen. The air in the chamber is then evacuated to a vacuum of 28 inches of Hg.

The macrocarrier is accelerated with a helium shock wave using a rupture membrane that bursts when the He pressure in the shock tube reaches 1000 psi.

Seven days after bombardment the tissue can be transferred to N6 medium that contains bialophos (5 mg per liter) and lacks casein or proline. The tissue continues to grow slowly on this medium. After an additional 2 weeks the tissue can be transferred to fresh N6 medium containing bialophos. After 6 weeks, areas of about 1 cm in diameter of actively growing callus can be identified on some of the plates containing the bialophos-supplemented medium. These calli may continue to grow when sub-cultured on the selective medium.

Plants can be regenerated from the transgenic callus by first transferring clusters of tissue to N6 medium supplemented with 0.2 mg per liter of 2,4-D. After two weeks the tissue can be transferred to regeneration medium (Fromm et al. (1990) *Bio/Technology* 8:833-839).

#### EXAMPLE 5

##### Expression of Recombinant DNA Constructs in Dicot Cells

A seed-specific expression cassette composed of the promoter and transcription terminator from the gene encoding the  $\beta$  subunit of the seed storage protein phaseolin from the bean *Phaseolus vulgaris* (Doyle et al. (1986) *J. Biol. Chem.* 261:9228-9238) can be used for expression of the instant polypeptides in transformed soybean. The phaseolin cassette includes about 500 nucleotides upstream (5') from the translation initiation codon and about 1650 nucleotides downstream (3') from the translation stop codon of phaseolin. Between the 5' and 3' regions are the unique restriction endonuclease sites NcoI (which includes the ATG translation initiation codon), SmaI, KpnI and XbaI. The entire cassette is flanked by HindIII sites.

The cDNA fragment of this gene may be generated by polymerase chain reaction (PCR) of the cDNA clone using appropriate oligonucleotide primers. Cloning sites can be incorporated into the oligonucleotides to provide proper orientation of the DNA fragment when inserted into the expression vector. Amplification is then performed as described above, and the isolated fragment is inserted into a pUC18 vector carrying the seed expression cassette.

Soybean embryos may then be transformed with the expression vector comprising sequences encoding the instant polypeptides. To induce somatic embryos, cotyledons, 3-5 mm in length dissected from surface sterilized, immature seeds of the soybean cultivar A2872, can be cultured in the light or dark at 26°C on an appropriate agar medium for 6-10 weeks. Somatic embryos which produce secondary embryos are then excised and placed into a suitable liquid medium.

After repeated selection for clusters of somatic embryos which multiplied as early, globular staged embryos, the suspensions are maintained as described below.

Soybean embryogenic suspension cultures can be maintained in 35 mL liquid media on a rotary shaker, 150 rpm, at 26°C with florescent lights on a 16:8 hour day/night schedule. Cultures are subcultured every two weeks by inoculating approximately 35 mg of tissue into 35 mL of liquid medium.

Soybean embryogenic suspension cultures may then be transformed by the method of particle gun bombardment (Klein et al. (1987) *Nature* (London) 327:70-73, U.S. Patent No. 4,945,050). A DuPont Biolistic™ PDS1000/HE instrument (helium retrofit) can be used for these transformations.

A selectable marker gene which can be used to facilitate soybean transformation is a chimeric gene composed of the 35S promoter from Cauliflower Mosaic Virus (Odell et al. (1985) *Nature* 313:810-812), the hygromycin phosphotransferase gene from plasmid pJR225 (from *E. coli*; Gritz et al. (1983) *Gene* 25:179-188) and the 3' region of the nopaline synthase gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*. The seed expression cassette comprising the phaseolin 5' region, the fragment encoding the instant polypeptides and the phaseolin 3' region can be isolated as a restriction fragment. This fragment can then be inserted into a unique restriction site of the vector carrying the marker gene.

To 50 µL of a 60 mg/mL 1 µm gold particle suspension is added (in order): 5 µL DNA (1 µg/µL), 20 µL spermidine (0.1 M), and 50 µL CaCl<sub>2</sub> (2.5 M). The particle preparation is then agitated for three minutes, spun in a microfuge for 10 seconds and the supernatant removed. The DNA-coated particles are then washed once in 400 µL 70% ethanol and resuspended in 40 µL of anhydrous ethanol. The DNA/particle suspension can be sonicated three times for one second each. Five µL of the DNA-coated gold particles are then loaded on each macro carrier disk.

Approximately 300-400 mg of a two-week-old suspension culture is placed in an empty 60x15 mm petri dish and the residual liquid removed from the tissue with a pipette. For each transformation experiment, approximately 5-10 plates of tissue are normally bombarded. Membrane rupture pressure is set at 1100 psi and the chamber is evacuated to a vacuum of 28 inches mercury. The tissue is placed approximately 3.5 inches away from the retaining screen and bombarded three times. Following bombardment, the tissue can be divided in half and placed back into liquid and cultured as described above.

Five to seven days post bombardment, the liquid media may be exchanged with fresh media, and eleven to twelve days post bombardment with fresh media containing 50 mg/mL hygromycin. This selective media can be refreshed weekly. Seven to eight weeks post bombardment, green, transformed tissue may be observed growing from untransformed, necrotic embryogenic clusters. Isolated green tissue is removed and inoculated into individual flasks to generate new, clonally propagated, transformed embryogenic suspension cultures. Each new line may be treated as an independent transformation event. These suspensions can then be subcultured and maintained as clusters of immature embryos or regenerated into whole plants by maturation and germination of individual somatic embryos.

### EXAMPLE 6

#### Expression of Recombinant DNA Constructs in Microbial Cells

The cDNAs encoding the instant polypeptides can be inserted into the T7 *E. coli* expression vector pBT430. This vector is a derivative of pET-3a (Rosenberg et al. (1987) *Gene* 56:125-135) which employs the bacteriophage T7 RNA polymerase/T7 promoter system. Plasmid pBT430 was constructed by first destroying the EcoRI and HindIII sites in pET-3a at their original positions. An oligonucleotide adaptor containing EcoRI and Hind III sites was inserted at the BamHI site of pET-3a. This created pET-3aM with additional unique cloning sites for insertion of genes into the expression vector. Then, the NdeI site at the position of translation initiation was converted to an NcoI site using oligonucleotide-directed mutagenesis. The DNA sequence of pET-3aM in this region, 5'-CATATGG, was converted to 5'-CCCATGG in pBT430.

Plasmid DNA containing a cDNA may be appropriately digested to release a nucleic acid fragment encoding the protein. This fragment may then be purified on a 1% low melting agarose gel. Buffer and agarose contain 10 µg/ml ethidium bromide for visualization of the DNA fragment. The fragment can then be purified from the agarose gel by digestion with GELase™ (Epicentre Technologies, Madison, WI) according to the manufacturer's instructions, ethanol precipitated, dried and resuspended in 20 µL of water. Appropriate oligonucleotide adapters may be ligated to the fragment using T4 DNA ligase (New England Biolabs (NEB), Beverly, MA). The fragment containing the ligated adapters can be purified from the excess adapters using low melting agarose as described above. The vector pBT430 is digested, dephosphorylated with alkaline phosphatase (NEB) and deproteinized with phenol/chloroform as described above. The prepared vector pBT430 and fragment can then be ligated at 16°C for 15 hours followed by transformation into



DH5 electrocompetent cells (GIBCO BRL). Transformants can be selected on agar plates containing LB media and 100 µg/mL ampicillin. Transformants containing the gene encoding the instant polypeptides are then screened for the correct orientation with respect to the T7 promoter by restriction enzyme analysis.

5 For high level expression, a plasmid clone with the cDNA insert in the correct orientation relative to the T7 promoter can be transformed into *E. coli* strain BL21(DE3) (Studier et al. (1986) *J. Mol. Biol.* 189:113-130). Cultures are grown in LB medium containing ampicillin (100 mg/L) at 25°C. At an optical density at 600 nm of approximately 1, IPTG (isopropylthio-β-galactoside, the inducer) can be  
10 added to a final concentration of 0.4 mM and incubation can be continued for 3 h at 25°. Cells are then harvested by centrifugation and re-suspended in 50 µL of 50 mM Tris-HCl at pH 8.0 containing 0.1 mM DTT and 0.2 mM phenyl methylsulfonyl fluoride. A small amount of 1 mm glass beads can be added and the mixture sonicated 3 times for about 5 seconds each time with a microprobe  
15 sonicator. The mixture is centrifuged and the protein concentration of the supernatant determined. One µg of protein from the soluble fraction of the culture can be separated by SDS-polyacrylamide gel electrophoresis. Gels can be observed for protein bands migrating at the expected molecular weight.

20 The activity of the lipoxygenase introduced into monocot cells (Example 4), dicot cells (Example 5) or microbial cells (Example 6) may be evaluated. Assays may be conducted under well known experimental conditions which permit optimal enzymatic activity of the lipoxygenase as presented by Grossman and Zakut (1979) *Methods Biochem. Anal.* 25:303-329.